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Full Length Article

Humoral Antibody Production Against *Salmonella typhi* in Frog (*Rana tigrina*)

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ABSTRACT

Studies on humoral antibody production using particulate antigens, *Salmonella typhi* were conducted. Data revealed that the use of *S. typhi* in both primary as well as in challenge produced higher anti-*S. typhi* antibodies as compared to that of the frogs, which received *S. typhi* either during primary or in the challenge. Moreover, in this case the use of adjuvant had enhancing effect.

Key Words: Humoral immunological memory; Frogs; Larval administration; Soluble and particulate antigens

INTRODUCTION

Amphibians are able to generate specific antibodies to a range of antigens. Different antigens elicit different antibody iso-types for example, antibacterial agglutinins involve IgM exclusively, whereas viruses and serum proteins initially elicit IgM production, which is later accompanied but not replaced by a switch to IgY production. A moderate affinity maturation of IgM is seen during an immune response. T-dependent and T-independent antigens have been categorized in *Xenopus* (Ruben, 1985; Ruben *et al*., 1986). In urodeles response to T-dependent antigens is relatively poor (Charlemagne & Tournefier, 1977).

An adjuvant is a substance, incorporated into or injected simultaneously with antigen, which potentiates the immune response (Balls, 1989). Adjuvants enhance the immune response, alter the nature of an immune response to a given immunogen or elicit an immune response by themselves (Pollara *et al.*, 1996). Adjuvants are of many types. Alum and other aluminum salts, vitamin A, certain endotoxins of bacteria and the Freund's complete and incomplete adjuvants. Adjuvants have several functions. They can change the non-immunogenic substances to immunogenic ones. They increase cell-mediated hypersensitivity. They can produce more effective protective immunity (Wang *et al.*, 2004). In the present study the use of FCA along with particulate antigens enhances the antibody production along with time.

MATERIALS AND METHODS

Animals and their maintenance. The frogs (*Rana tigrina*) were collected from various localities of Lahore. The average body weight (g) was 98.8 ± 7.67 and average snout-vent length (cm) was 10.6 ± 0.06. During experiments, frogs were maintained in plastic tubs of 58 cm diameter and of 26 cm depth containing approximately $^1/_3$ standing dechlorinated water.

Particulate antigen. *Salmonella typhi* was used as particulate antigen. The bacterial suspension was purchased from market, available as Tab Vaccine (Swiss serum & Vaccine). The bacterial suspension contained inactivated 5 x 10^9 cells mL^{-1}. This suspension was diluted to 5 x 10^8 cells mL^{-1} with 0.85% saline and injected at the rate of 5 µL g^{-1} body weight in a way that each animal received 10^6 cells g^{-1} body weight.

Challenge injections. All challenge doses were administered in adjuvant in order to ensure that the dose was potentially immunogenic. The dose was 25 µg g^{-1} body weight for soluble antigens, delivered at a rate of 0.0005 mL g^{-1} body weight, whereas *S. typhi* vaccine dose was 10^8 formalin-milled cells g^{-1} body weight.

Anaesthesia and Post-Mortem Procedure

Anaesthesia. Chloroform (BDH Chemical Poole, England) was used as an anaesthetic. The frogs were put into a closed glass container with strong vapours of chloroform and were removed immediately after they fell unconscious.

Blood collection. Adult frogs were bled aseptically from the femoral vein. Lower leg was swabbed with 70% alcohol, skin was cut and vein was nicked with a sterile scalpel blade. The flowing blood was collected using 2 mL disposable syringe, then transferred to a centrifuge tube. Frogs that were too small to be bled in this manner were anaesthetized and opened to expose the heart. Blood was then collected directly from the ventricle using a capillary tube. The animals were then killed.

Serum preparation. The blood of adult frogs was collected in 10 mL centrifuge tubes (Sterilin, U.K.) and that from small animals in 1.5 mL micro-capped centrifuge tubes (Hughes & Hughes, U.K.). The blood was left to clot (1 to 2

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h) at room temperature and then kept overnight at 4°C for clot retraction. The 10 mL centrifuge tubes were then centrifuged at 600 g (3000-3500 rpm) for 5 min and 1.5 mL tubes centrifuged in a one speed micro-centrifuge for 2 - 3 min. Serum that was to be used as the diluent in passive haemagglutination tests was pooled, heat-inactivated at 56°C for 30 min for frogs and lots in micro-centrifuge tubes and stored at 20°C. Test sera were always used fresh, because freezing reduces the antibody levels (Rio & Recco, 1971).

Bacterial agglutination. Antibody titres against *S. typhi* were tested using a modified form of agglutination technique (Secombes & Manning, 1980). In the test, which was carried out in micro titer plates (Sterlin, U.K.), 50 µL of 0.85% saline was added in rows A then 50 µL of normal serum was added in well No. 1 of row A (first row) and then serially diluted by two fold steps up to well No. 12. From row B to H 50 µL of test serum was serially diluted two fold, up to well No. 12 in each column and 50 µL of 10^6 cell mL^{-1} saline of *S. typhi* was added to each well. The plate was then gently agitated and sealed with tape. It was kept at room temperature for 3 h and then overnight at 4°C before reading the plate. End points (last well showing complete agglutination) were noted by looking at the plate from below with the naked eye or under a dissecting microscope.

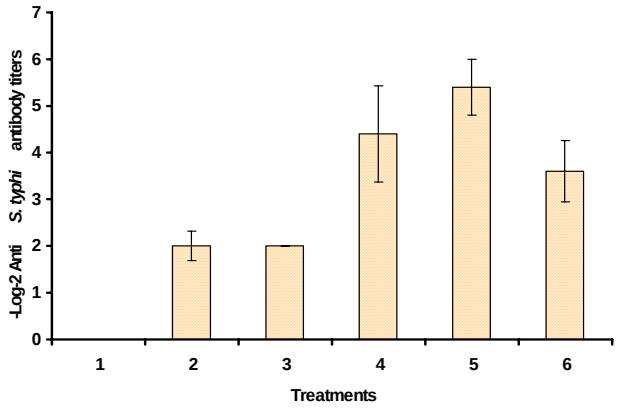
RESULTS

Group I. The frogs were given primary treatment of *S. typhi* in saline or in adjuvant, while some groups received only saline instead of antigen. The secondary treatment of *S. typhi* in saline or in adjuvant was given four weeks after the primary injection. The frogs were sacrificed two weeks after the secondary treatment for their humoral antibody production and produced variable antibody titers.

The frogs that did not receive any antigen did not show any naturally existing anti-*S. typhi* antibodies in their blood (0.0 ± 0.0). Frogs that received saline as primary treatment and *S. typhi* in saline or in adjuvant as challenge produced 2.0 ± 0.31 – log 2 and 2.0 ± 0.0 – log 2 number of wells antibody titers, respectively. *S. typhi* in saline as primary as well as secondary treatment produced 4.4 ± 1.03 – log 2 antibody titers, while those which received *S. typhi* in adjuvant as challenge, produced 5.4 ± 0.61 – log 2 antibody titer whereas, *S. typhi* in adjuvant as primary as well as secondary treatment produced 3.6 ± 0.67 – log 2 number of wells antibody titers (Fig. 1).

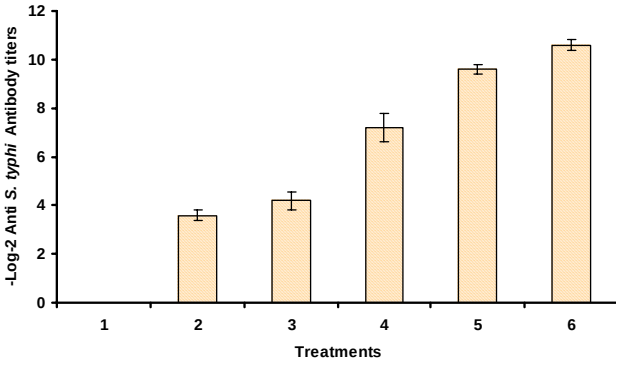
These results show that primary treatment of saline followed by challenge of *S. typhi* in saline or in adjuvant (one dose of *S. typhi*), produced significantly lower antibody titers as compared to those frogs that received *S. typhi* in saline as primary and *S. typhi* in saline or in adjuvant as secondary treatment (two doses of *S. typhi*). *S. typhi* in adjuvant as primary as well as secondary produced high number of antibody titers when compared with those that received one dose of *S. typhi* in saline only and other dose of

Fig. 1. Humoral antibody production against particulate antigen *S. typhi*, two weeks after the secondary treatment (Group-I)



	Primary	Secondary
1.	Saline	Saline
2.	Saline	*S. typhi* in saline
3.	Saline	*S. typhi* in adjuvant
4.	*S. typhi* in saline	*S. typhi* in saline
5.	*S. typhi* in saline	*S. typhi* in adjuvant
6.	*S. typhi* in adjuvant	*S. typhi* in adjuvant

Fig. 2. Humoral antibody production against particulate antigen *S. typhi*, four weeks after the secondary treatment (Group-II)



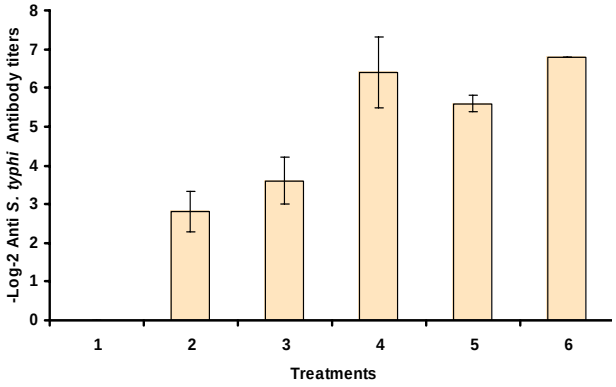
	Primary	Secondary
1.	Saline	Saline
2.	Saline	*S. typhi* in saline
3.	Saline	*S. typhi* in adjuvant
4.	*S. typhi* in saline	*S. typhi* in saline
5.	*S. typhi* in saline	*S. typhi* in adjuvant
6.	*S. typhi* in adjuvant	*S. typhi* in adjuvant

S. typhi in saline or in adjuvant. So in case of *S. typhi* it was evident that the adjuvant enhanced the response.

Group II. The frogs were given primary treatment of *S. typhi* in saline or in adjuvant and second injection of *S. typhi* in saline or in adjuvant was given four weeks after the primary treatment. These frogs were sacrificed four weeks after the challenge injection for their humoral antibody production (Fig. 2).

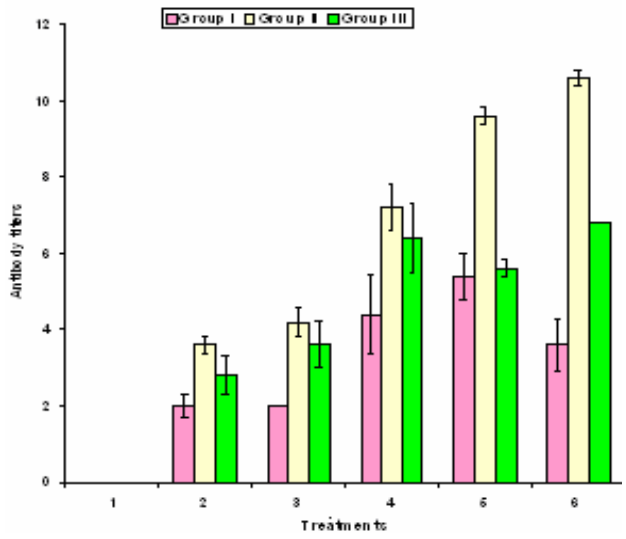
Saline as primary treatment and *S. typhi* in saline as

Fig. 3. Humoral antibody production against particulate antigen *S. typhi*, six weeks after the secondary treatment (Group-III)



	Primary	Secondary
1	Saline	Saline
2.	Saline	*S. typhi* in saline
3.	Saline	*S. typhi* in adjuvant
4.	*S. typhi* in saline	*S. typhi* in saline
5.	*S. typhi* in saline	*S. typhi* in adjuvant
6.	*S. typhi* in adjuvant	*S. typhi* in adjuvant

Fig. 4. Comparison of *S. typhi* between Groups I, II and III.



secondary treatment produced 3.6 ± 0.24 – log 2 number of wells antibody titers, while those frogs that received *S. typhi* in adjuvant in secondary treatment produced 4.2 ± 0.37 – log 2 number of wells antibody titers. At the same time, *S. typhi* in saline as primary and in saline or in adjuvant as secondary treatment produced significantly different levels of antibody titers 7.2 ± 0.59 – log 2 and 9.6 ± 0.22 – log 2 number of wells anti-*S. typhi* antibody titres, respectively. Similarly, *S. typhi* in adjuvant both in primary as well as in secondary treatment produced highest antibody levels in comparison with other animals 10.6 ± 0.22 – log 2 number of wells antibody titers. These results show marked differences between various groups of primary and secondary treatments only one dose of *S. typhi* in secondary treatment produced significantly lower antibody titers when compared with results of the frogs that received *S. typhi* both in primary as well as in secondary treatment (i.e., received two doses of *S. typhi*). Use of adjuvant in this group showed higher levels of antibody (Fig. 2).

Group III. Frogs were given treatment of *S. typhi* in saline or in adjuvant as primary treatment, while some animals received only saline. Secondary treatment of *S. typhi* was given either in saline or in adjuvant four weeks after primary treatment. These frogs were sacrificed six weeks after the secondary treatment for humoral antibody production (Fig. 3). Results of the experiments show that saline only as primary treatment and *S. typhi* in saline or in adjuvant as secondary treatment produced 2.8 ± 0.52 – log 2 and 3.6 ± 0.61 – log 2 number of wells of antibody titers, respectively. Frogs that received *S. typhi* as primary injection and *S. typhi* in saline or in adjuvant as secondary treatment produced 6.4 ± 0.9 – log 2 and 5.6 ± 0.22 – log 2 number of wells, respectively. These figures are significantly higher than those animals, which did not receive *S. typhi* as primary treatment. *S. typhi* in combination with adjuvant at primary and secondary levels of treatment produced 6.8 ± 0.0 – log 2 numbers of wells of antibody titers. Levels of antibody titers were in the same range in case of *S. typhi* in saline primary and secondary doses as well as in *S. typhi* in adjuvant in both doses (Fig. 3).

Comparison of *S. typhi* two weeks after, four weeks after and six weeks after showed that all three groups resulted in higher titers of antibody production after 4 weeks of administration of challenge injections (Fig. 4).

DISCUSSION

Results of the study suggest that *Rana tigrina* is capable to mount good primary immune response. Frogs that received single injection of antigen produced considerable amount of antibodies two weeks after the injection of antigens. The peak immune response was obtained after four weeks and then there was a decline in the antibody titres as was measured six weeks after. It is well documented that other amphibians are able to produce antibodies after receiving single dose of cellular or particulate antigen. When a particulate antigen, *Salmonella adelaide flagella*, was administered to *Bufo marinus*, it induced humoral immune response similar to that of mice (Diener & Marchalonis, 1970). *Bufo marinus* also produced good primary responses upon stimulation with bacteriophage F2 in FCA (Lin *et al*., 1971; Du-Pasquier, 1982; Du-Pasquier *et al.*, 1994). Similarly, *Ambystoma maxicanum* produced immune responses when injected with horse red blood cells (Charlemagne & Tournefier, 1977).

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